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# US ARMY MEDICAL RESEARCH LABORATORY

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REPORT NO. 452

## ELECTROPHORETIC MOBILITY AND ANTIGENICITY OF X-IRRADIATED HUMAN SERUM GAMMA GLOBULIN

Anthony J. Luzzio, Ph. D.

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Effects of Ionizing Ra. on Immune Mechanisms  
Task 01  
Biological and Medical Aspects of Ionizing Radiation  
USAMRL Project No. 6X64-14-001

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Report No. 452  
USAMRL Project No. 6X64-14-001-01

ABSTRACT

ELECTROPHORETIC MOBILITY AND ANTIGENICITY  
OF X-IRRADIATED HUMAN SERUM GAMMA GLOBULIN

OBJECT

To determine the effects of ionizing rays on the electrophoretic mobility and antigenic specificity of human gamma globulin.

RESULTS AND CONCLUSIONS

Exposure of HSGG to various doses of X-rays results in the production of molecules electrophoretically distinct from the parent protein. The evidence that structural changes take place is given support by the finding that the antigenic specificity of the new derivative is far removed from that of the starting material. It is suggested that HSGG denatured by ionizing rays has definite and controllable characteristics.

APPROVED:

Adolph T. Krebs  
ADOLPH T. KREBS  
Director, Radiobiology Division

APPROVED:

Floyd A. Odell  
FLOYD A. ODELL, Ph. D.  
Technical Director of Research

APPROVED:

Harold W. Glascock Jr.  
HAROLD W. GLASCOCK JR.  
Colonel, Medical Corps  
Commanding

## ELECTROPHORETIC MOBILITY AND ANTIGENICITY OF X-IRRADIATED HUMAN SERUM GAMMA GLOBULIN

### I. INTRODUCTION

Since Hardy's report (1) on protein denaturation by radium bromide the effects of ionizing radiation on aqueous solutions of proteins have been extensively studied. Fernau and Spiegel-Adolph (2), Pedersen (3), Sanigar, Krejci, and Kraemer (4) were among the first to study changes in various physico-chemical properties of proteins. Since then a number of investigators, Arnow (5), Fricke (6), Barron and Flood (7), Barron and Johnson (8), Ebert and Swallow (9), have reported changes in the measurable properties of irradiated proteins in solution. A large number of these studies are concerned with measurements of viscosity, solubility, coagulability, optical density, and deactivation of specific enzymatic properties. From an experimental, as well as from a physiological viewpoint, an important result of the alterations produced by ionizing radiation is the loss or modification of specific immunological properties. That antibody specificity can be modified has been demonstrated by Chambers and Russ (10), Lawrence and Graikoski (11), and Luzzio (12, 13). However, the number of reports dealing with specific antigenic changes of serum proteins following irradiation is relatively few. Results which have been reported by Muntz, Barron, and Prosser (14), Fischer, Magee, and Coulter (15), and Winkler and Paschke (16) suggest alterations in the molecular structure of certain serum proteins in the irradiated animal. In view of these results questions concerning possible modifications in the antigenicity of radiation-altered serum proteins are extremely interesting problems of fundamental importance. The present report concerns experiments designed to determine the effect of X-irradiation on the electrophoretic mobility of human serum gamma globulin (HSGG), and to characterize the observed changes immunologically.

### II. MATERIALS AND METHODS

HSGG and human serum albumin (HSA), obtained from the American Red Cross, Washington, D. C., were studied. The protein fractions were prepared by Cohn fractionation and reported to be better than 97 per cent pure by electrophoretic methods. The homogeneity of the protein was determined by starch block and paper strip electrophoresis. Only those solutions which migrated in a single peak were used for our experiments.

For X-irradiation eight two ml samples of protein adjusted to pH 7.5 were placed in open planchets and exposed to various doses. X-rays were delivered by a General Electric Maxitron unit operated at 250 kvp, 30 ma, 4.75 mm Be inherent filtration and a target to protein surface distance of 12.5 cm. Dose-rate measurements were made using chemical dosimetry methods under identical experimental irradiation conditions. A dose rate of 30,515 r/min was determined by measuring spectrophotometrically the reduction of ceric sulphate solution (17). Samples of irradiated protein were retained for paper and starch electrophoresis, and for antigen--antibody reactions.

Paper electrophoresis was carried out at room temperature with an LKB apparatus; 100 v and 4.0 ma being applied to the electrodes. Schleicher and Schuell paper was used with a veronal (diethylbarbituric acid) sodium acetate buffer at pH 8.6, ionic strength of 0.125. Samples were applied to the paper strips by means of an applicator to which 12  $\mu$ l of protein solution had been delivered with an ultra micropipette. One control unirradiated and seven samples which had received various doses of X-rays were run in parallel. After 16 hours the strips were removed and dried for 30 minutes in an oven set at 107°C. The strips were stained for six hours in bromphenol blue dye at room temperature. This was followed by two washes in five per cent acetic acid. Fixing was for six minutes in acetic acid--sodium acetate fixative. Subsequent to blotting and drying at 107°C electrophoretic patterns were obtained by scanning in a Spinco Model R 110-115v, 60 cycle analyzer.

Separation of electrophoretic components was also carried out by starch-block electrophoresis. The apparatus used differs very little from that described by Kunkel and Slater (18). Two ml of gamma globulin, containing 45 mg protein per ml, were mixed with dry potato starch so that the thin slurry which formed could be poured into a rectangular slit cut transversely 8 cm from one end of a starch block 46 x 48 cm and 0.5 cm thick. The starch block was equilibrated with phosphate buffer pH 7.7 ionic strength 0.1 for two hours at 5°C after which an electromotive force of 115v, 30 ma was applied. At the end of 72 hours the starch block was allowed to dry partially and then partitioned into 1 cm segments. Each segment was eluted with phosphate buffer and the eluates tested for protein content by the biuret method used by Cornall, Bardawill, and David (19). Antigenic specificity of pooled eluates was determined by reactions with unirradiated and X-irradiated rabbit anti-HSGG.

New Zealand albino rabbits were used for immune serum production. The animals received a first subcutaneous inoculation of 22 mg of antigen

followed by a second and third dose of 45 mg administered intravenously on the third and fifth days. The antigens used were unirradiated HSGG and HSGG exposed to  $5.49 \times 10^6$  r. After seven days rest the animals were bled by cardiac puncture under nembutal anesthesia. The blood was allowed to clot at 5°C and the serums were collected by decantation and centrifugation. Blood was collected every third week with a booster inoculation consisting of 45 mg of antigen administered one week after each bleeding. The serums collected were stored at -5°C in small quantities and thawed for use as needed.

Precipitin titers were determined by the serial twofold dilution method. The technique employed consisted of layering 0.1 ml of antigen dilution on 0.1 ml of non-irradiated and X-irradiated rabbit anti-HSGG. The first tube of each titration series contained 22 mg of antigen per ml. The tubes were placed at room temperature for the first hour and 5°C for the second hour. Readings were taken at 15-minute intervals. The highest dilution which demonstrated a definite precipitin at the antigen - antiserum interphase was recorded as the end point of the titration.

Two methods were used for preparing antiserum specific for radiation-altered gamma globulin. The conventional routine method of antibody absorption, and another technique based on earlier work (12, 13), which indicates antibodies in a polyvalent antiserum may be inactivated selectively by utilizing X-rays at the proper dose level.

### III. RESULTS

In Figure 1, page 4, is reproduced a series of electrophoretic patterns typical for HSGG subsequent to X-ray exposure. A total of 200 exposures were made. It is apparent that progressive increases in dose resulted in a relative increase of molecules different in electrophoretic mobility than the original globulin. These molecules begin to form after  $3.66 \times 10^6$  r with two well defined peaks clearly resolved after exposure of the protein to  $5.49 \times 10^6$  r. It is interesting to note (table 1, page 5) that this effect could not be produced when HSGG was exposed in concentrations greater than 54.4 mg/ml. This illustrates a dilution dependency very well. No change in the electrophoretic pattern of human serum albumin was effected when exposed to the same doses of X-rays and in similar concentrations as HSGG.

Figure 2, page 6, is the typical pattern obtained when starch block electrophoresis was used as a preparative method for separating radiation altered HSGG from the parent protein. After exposure to 5.49



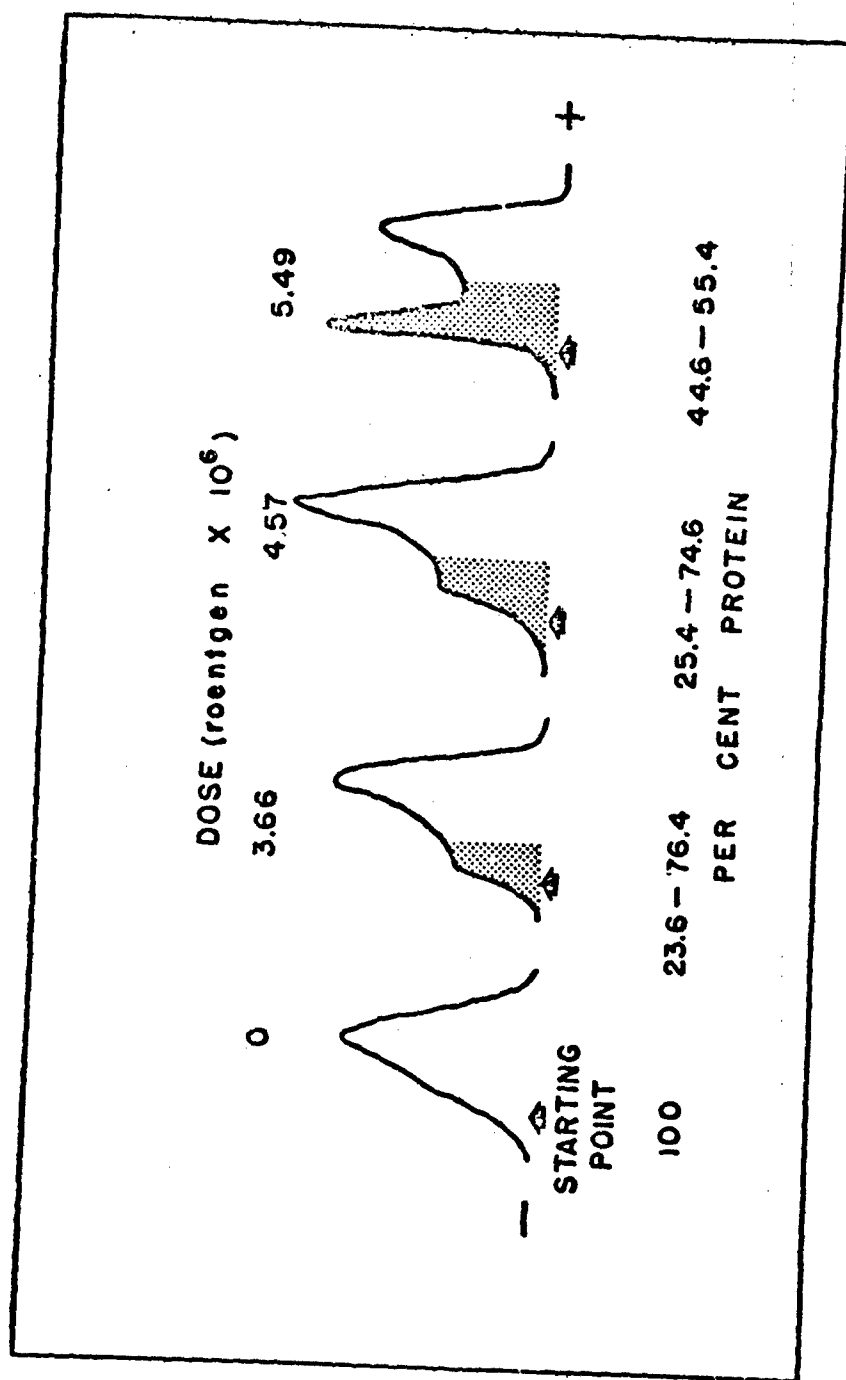


Fig. 1. Paper electrophoresis of X-irradiated HSCG (45 mg/ml). Shaded areas represent slower moving component after irradiation.

TABLE 1

THE EFFECT OF PROTEIN CONCENTRATION ON X-IRRADIATION OF  
HUMAN GAMMA GLOBULIN

Dose $\times 10^6$	Pre-Irrad Protein Mg/ml	Electrophoretic Peaks		Post-Irrad Protein Mg/ml
		Non-migrating Mg Prot./ml	Migrating Mg Prot./ml	
0	157.0	157.0	-	-
1.83	-	157.0	-	157.0
4.57	-	157.0	-	157.0
0	54.4	54.4	-	-
1.83	-	54.4	-	54.4
5.49	-	54.4	-	54.4
0	40.0	40.0	-	-
1.83	-	8.5	31.5	40.0
2.74	-	6.4	33.6	40.0
3.66	-	6.9	33.1	40.0
4.57	-	3.8	36.2	40.0
5.49	-	14.0	26.0	40.0
0	19.5	19.5	-	-
1.83	-	19.5	-	-
2.74	-	4.3	15.2	19.5
3.66	-	7.8	11.7	19.5
4.57	-	10.7	8.8	19.5
5.49	-	6.8	12.7	19.5

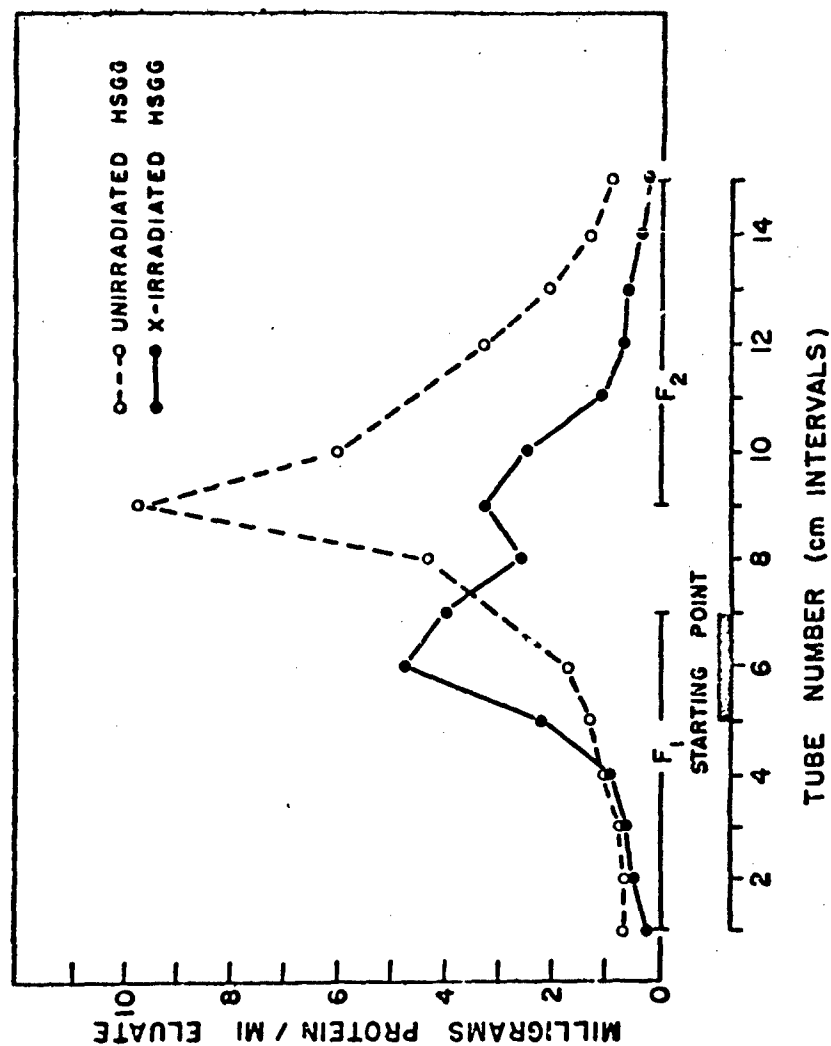


Fig. 2. Starch block electrophoresis of unirradiated and X-irradiated ( $5.49 \times 10^6$  r) HSGG. F<sub>1</sub> fraction giving positive precipitin test with only rabbit anti-X-irradiated HSGG. Fraction F<sub>2</sub> corresponds serologically only with original HSGG.

$\times 10^6$  r two well defined peaks were demonstrated. The pattern did not differ significantly from that obtained with paper electrophoresis methods.

At this stage interest was directed to the nature of the antigenicity of each of the fractions separated by starch electrophoresis, Figure 2. Precipitin tests with antiserum prepared by immunizing rabbits with HSGG exposed to  $5.49 \times 10^6$  r showed high titers with both unirradiated and X-irradiated HSGG. Absorption of this polyvalent antiserum with HSGG resulted in a serum which produced a precipitate only with X-irradiated HSGG. However, the titers of serums prepared by this method were extremely low. Table 2 summarizes the typical results obtained when pooled rabbit anti-X-irradiated HSGG from three rabbits was exposed to various doses of X-rays and titrated with unirradiated and the homologous X-irradiated antigen. A total of 24 rabbits was used. Each pooled serum was exposed and titrated in duplicate. Similar results were observed with antiserum from 12 individual rabbits bled at various intervals during the immunization schedule. The data show that X-irradiating antisera with certain doses of X-rays resulted in the reduction or complete modification of combining sites for unaltered HSGG. The reaction of antibodies specific for X-irradiated HSGG antigen remained relatively stable in comparison with the reaction between unirradiated antigens at the same dose levels. However, it is interesting to note that this phenomenon occurred only in serums collected early in the immunization and bleeding schedule of the experimental rabbits. Later bleedings produced serums indicating the titer of combining sites for unaltered HSGG were not as markedly affected by X-irradiation.

One striking observation made was that after certain X-ray dose levels the titer of an antiserum for X-irradiated HSGG was decreased and subsequent to the next increase in dose level was increased. In one instance the titer increase was eightfold over the control. A further increase in dose resulted in complete inactivity, table 2.

Antiserum highly specific for X-ray altered HSGG prepared by the method described above, Luzzio (12, 13), gave precipitates only with the slow moving starch electrophoresis fraction, Figure 2,  $F_1$ . The more mobile fraction corresponded serologically only to the parent HSGG, Figure 2,  $F_2$ .

TABLE 2

## TITRATION OF X-IRRADIATION RABBIT ANTI-X-IRRADIATED HSGG

HSGG Antigen	Dose (roentgens $\times 10^6$ )						Bleeding
	0	0.91	1.83	2.74	3.66	4.56	5.49
*UN-XI ** XI	1-2048 1-2048	1-512 1-512	1-512 1-256	1-512 1-128	1-512 1-128	1-512 1-16, 384	0 1-1096
UN-XI XI	1-1024 1-512	1-2048 1-256	1-512 1-256	1-1024 1-256	1-256 1-128	1-256 1-128	1-64 1-128
UN-XI XI	1-256 1-128	1-1024 1-256	1-1024 1-128	1-512 1-64	0 1-2048	0 0	0 0
UN-XI XI	1-16, 384 1-512	1-16, 384 1-512	1-16, 384 1-256	1-16, 384 1-32	0 0	0 0	0 0
UN-XI XI	1-512 1-512	1-2048 1-512	1-2048 1-128	1-256 1-64	1-256 1-256	1-1024 1-128	1-16 1-16
UN-XI XI	1-16, 384 1-1024	1-16, 384 1-512	1-16, 384 1-1024	1-16, 384 1-512	1-1024 1-32	1-1024 1-256	1-1024 1-128

\*UN-XI Un-X-Irradiated HSGG

\*\*XI X-Irradiated HSGG ( $5.49 \times 10^6$  r)

#### IV. DISCUSSION

It is well known that total serum gamma globulin represents a vast spectrum of proteins with similar but slightly different mobilities and sedimentation rates. It is not the intent of this writer to discuss which of the molecular species is affected by X-irradiation, or to attempt to give a complete explanation on the nature of the physical and chemical changes that occur. By virtue of its heterogeneity, in structure and function, this would be a task as complex to perform at this time as the protein itself. For interesting views the writer gives reference to Fricke (6), Svedburg and Brohuit (20, 21), Spiegel, A. (22), and Barron et al (23). However, on the basis of the work cited and the data reported herein, it seems appropriate to make several speculations.

The experimental results which have been reported here have significance for studies concerning protein denaturation by ionizing rays. The appearance of a second electrophoretic peak is evidence that definite structural changes take place in HSGG during X-irradiation. This evidence is given additional support by the finding that the antigenic specificity of the new derivative is far removed from that of the starting material. In the light of modern concepts concerning protein structure, it appears that in the process of denaturation by X-irradiation an uncoiling and separation of the peptide chains into long extended forms occurs. It seems likely that new combining sites become dominant in the denatured HSGG which cause the stimulation of antibodies of a different specificity. The finding that the specificity of denatured HSGG is different from that of the starting material, and reproducible, shows that the denatured state of this protein is not disordered. Further, it shows that denatured HSGG has definite and controllable characteristics. This is reflected in a stimulus toward the formation of antibodies as specific as those following the injection of native HSGG. The present report supports an earlier work concerning the selective inactivation of antibody combining sites by certain doses of ionizing rays (13, 14).

The observation that after certain X-ray doses the titer of an anti-serum to an antigen may significantly exceed that of the control serum has also been made in experiments using serums of specificities other than those discussed here, Luzzio (24). The significant increases in titer cannot be accounted for in terms of evaporation or errors in technique. A simple explanation is that at certain doses surface groups are modified and the titer falls. Higher doses serve only to lengthen the protein helix, and expose reactive groups which were previously inaccessible.

With continuous immunization antibody combining sites for unaltered HSGG were not as markedly affected as in earlier serums when exposed to the same doses of X-rays. At this time the phenomenon responsible for this observation may be speculated on in terms of earlier work by Kekwick and Record (25), Kekwick, *et al* (26), and Relyveld and Raynaud (27). These workers have shown that horse antitoxic serums contain two varieties of antitoxin when examined by electrophoresis. The proportions of these changes with continuous immunization.

## V. SUMMARY

Exposure of HSGG to various doses of X-rays results in the production of molecules electrophoretically distinct from the parent protein. The evidence that structural changes take place is given support by the finding that the antigenic specificity of the new derivative is far removed from that of the starting material. It is suggested that HSGG denatured by ionizing rays has definite and controllable characteristics.

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